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THE CONSEQUENCES OF PROSTANOID SYNTHESIS AND RELEASE BY HUMAN PERIPHERAL BLOOD MONOCYTES ON IMMUNE FUNCTION AND CELL PROLIFERATION

The Ohio State University

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Ph.D. 1985
THE CONSEQUENCES OF PROSTANOID SYNTHESIS
AND RELEASE BY HUMAN PERIPHERAL BLOOD
MONOCYTES ON IMMUNE FUNCTION AND
CELL PROLIFERATION

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
The Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Jenifer Ann Lindsey

*****

The Ohio State University

1985

Reading Committee: Approved by
Dr. David G. Cornwell
Dr. Howard Sprecher
Dr. Rao V. Panganamala

Department of Physiological Chemistry
Dedicated in the memory of Clyde G. Corle
a scholar, an educator, a grandfather
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VITA

BIRTHDATE: December 4, 1958

PLACE OF BIRTH: Greenville, Ohio

RESIDENCE: 2029 Heathcliff Dr. Apt. 3-A
Columbus, Ohio 43209

EDUCATION
The Pennsylvania State University--B.S., 1981, (Biochemistry)
The Ohio State University--Ph.D., 1985, (Physiological Chemistry)

AWARDS:
The Helen L. Wikoff Award—for excellence in graduate student research—The Ohio State University, 1984

PROFESSIONAL EXPERIENCE:
Research Assistant—The Department of Physiological Chemistry
The Ohio State University, 1983-1985

Teaching Assistant—The Department of Physiological Chemistry
The Ohio State University, 1981-1983

SOCIETIES AND ORGANIZATIONS:
Phi Kappa Phi
Sigma Delta Upsilon
Graduate Student Council

PUBLICATIONS:

1983

Fatty Acid Metabolism and Cell Proliferation. IV. Effect of Prostanoid Biosynthesis from Endogenous Fatty Acid Release with Cyclosporin A. J.A. Lindsey, N. Morisaki, J.M. Stitts, R.A. Zager and D.G. Cornwell. Lipids, 18 (8), pp. 566-569.

1984


In Press


The Impaired Ability of Human Monocytes to Stimulate Autologous and Allogeneic Mixed Lymphocyte Reactions After Exposure to Cyclosporine A: Associate Alterations of HLA-DR Expression and Physical Characteristics of Monocytes. R.L. Whisler, J.A. Lindsey, K.V.W. Procter, Y.G. Newhouse and D.G. Cornwell. Transplantation.

ABSTRACTS:


Role of Prostaglandins in Promotion of Smooth Muscle Cell Proliferation by Monocyte-Conditioned Media. FASEB, April, 1985
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>20:4(n-6)</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>ALS</td>
<td>Antilymphocyte serum</td>
</tr>
<tr>
<td>AZA</td>
<td>Azathioprine</td>
</tr>
<tr>
<td>B-cell</td>
<td>B-lymphocyte</td>
</tr>
<tr>
<td>CSA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FMF</td>
<td>Flow microcytofluorometry</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigens</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
</tr>
<tr>
<td>IM</td>
<td>Indomethacin</td>
</tr>
<tr>
<td>LAF</td>
<td>Lymphocyte Activating Factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MPC</td>
<td>Mononuclear Phagocytic Cells</td>
</tr>
<tr>
<td>MØ</td>
<td>Monocyte/Macrophage</td>
</tr>
<tr>
<td>MØM</td>
<td>Monocyte-conditioned media</td>
</tr>
<tr>
<td>MØDGF</td>
<td>Monocyte Derived Growth Factor</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutin</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative of tuberculin</td>
</tr>
<tr>
<td>P</td>
<td>Prednisone</td>
</tr>
<tr>
<td>SBSS</td>
<td>Seligmann's balanced salt solution</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acids</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>T-Ag</td>
<td>Transplant antigen</td>
</tr>
<tr>
<td>T-cell</td>
<td>T-lymphocyte</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T-lymphocyte</td>
</tr>
<tr>
<td>Tp</td>
<td>Precursor T-lymphocyte</td>
</tr>
<tr>
<td>Ts</td>
<td>Suppressor T-lymphocyte</td>
</tr>
<tr>
<td>TDGF</td>
<td>T-lymphocyte Derived Growth Factor</td>
</tr>
</tbody>
</table>
INTRODUCTION

Mononuclear phagocytic cells, vital components of the immune-surveillance system, act as a constant defense against the intrusion of harmful antigenic compounds or organisms. In certain instances however, the presence and subsequent activation of these cells leads to detrimental and destructive events. Two examples of this are the involvement of these cells in: rejection of organ transplants and promotion of atherosclerotic lesion development.

Following organ transplantation, monocyte/macrophage cells (MØ) invade the area and trigger events which ultimately result in formation of cytotoxic T-lymphocytes, the effector cells of transplant rejection. MØ mediate responsiveness in two ways; by the release of soluble products, such as prostaglandins and expression of HLA-DR antigens, cell surface glycoproteins coded by the histocompatibility complex which are recognized by lymphocyte receptors. Marked increases in prostaglandins and decreases in HLA-DR expression by MØ coincide with suppression of rejection of organ transplants. Cyclosporine A, an immunosuppressant successfully used to prevent graft rejection, specifically inhibits T-lymphocyte responses by an as yet unknown mode of action. This agent does stimulate prostanoid synthesis in non-immunocompetent cells. This
dissertation examines the changes in prostanoid synthesis and HLA-DR
expression by Cyclosporine-treated human peripheral blood monocytes.
Effects on these phenomena might explain, in part, the mechanism of
action of this potent immunosuppressant.

Smooth muscle cell proliferation within the intima of the aorta is
involved in developing atherosclerotic lesions. MØ potentiate this
process by releasing MØ derived growth factor(s). Increased
prostanoid synthesis and release is also associated with increased
smooth muscle cell growth. The second portion of this dissertation
investigates the ability of human peripheral blood MØ and human
peripheral blood MØ conditioned media to promote prostanoid synthesis
and growth of aortal smooth muscle cells.
GENERAL CONCEPTS

TERMINOLOGY

In order to determine the validity of the purpose and conclusions of this dissertation, a general understanding of a few current concepts of basic immunology is needed. Thus, a very general overview of immunity is initially presented. This information was assimilated from various texts (Sites et al., 1982; Kimbal, 1983; Stansfield, 1981).

Immunity has been divided into two general classifications; Humoral Immunity, which includes responses involving antibody and complement production, and Cell-Mediated Immunity, which includes responses carried out directly by immunocompetent cells in the absence of antibody and complement. The major cellular components include; B-lymphocytes (B-cells), T-lymphocytes (T-cells) and monocyte/macrophage (MØ) cells. B-cells are responsible for antibody production and are sub-divided according to the antibody produced. T-cells are mainly responsible for cell mediated immune responses and are subdivided according to functions as either: regulatory cells [T helper (Th) and T suppressor (Ts)] or effector cells [T cytotoxic (Tc)]. MØ initiate, propagate and terminate the immune responses through various accessory functions and phagocytosis.
Basic terminology of transplantation begins with these classifications of organ transplants or grafts: autograft - transplantation of self to self; syngrafts or isograft - exchange of tissue between genetically identical individuals; allografts - exchange of tissue between genetically different individuals within the same species and xenografts - grafts between individuals of two different species. Autografts and syngrafts are normally accepted by the organ recipient without rejection. While allografts and xenografts are always rejected in the absence of immunosuppressive agents.

HLA COMPLEX AND ANTIGENS

Rejection of a transplanted organ, simply stated, is due to an immune response triggered by the presence of "foreign" antigens on the organ. These antigens; as well as "self" antigens on monocytes, T-cells and B cells, are coded by the Major Histocompatibility Complex (MHC) and in Man are called Human Leukocyte Antigens (HLA). MHC is often referred to as the HLA complex.

The HLA complex is located on the short arm of chromosome 6 and is approximately 0.5 centimorgins long. There are five major loci within this area; HLA-A, HLA-B, HLA-C, HLA-D and HLA-DR (D-related). Each locus is further subdivided by alternate forms of a gene (alleles), which are numbered. The products determined by the various alleles are the cell surface glycoproteins called HLA antigens. Because there are multiple alleles at each loci the HLA system is extremely polymorphic. Therefore, there are numerable possibilities for HLA antigens. A
complete listing of recognized HLA antigens according to the HLA Nomenclature Committee is shown in Table 1.

HLA antigens have been subdivided into two categories, Class I and Class II antigens, according to structure, function and tissue distribution. Class I antigens; HLA-A, HLA-B and HLA-C, are found on all cells. These antigens function as cell markers which identify the genetic origin of the cell. Class I antigens on the cell surface of an allograft, signal to the immunocompetent cells of the host, the presence of an "intruder". In turn, these antigens serve as the target molecules for the cytotoxic T-cells of the host. Class II antigens, HLA-D and HLA-DR are found mainly on resting T-cells, activated T-cells, MØ's and B-cells. These antigens are vital components of the intercellular communication that occurs among the immunocompetent cells (Selwood and Hedges, 1978; Schwartz, 1982).

CELLULAR EVENTS OF ALLOGRAFT REJECTION

A number of effector mechanisms may be ultimately involved in rejection of a transplanted organ (Mason, 1983). However, allograft rejection is considered to be primarily a cell mediated immune response initiated by presentation of "foreign" allograft antigens (T-Ag) by MØ to a precursor T-cell (Tp) (Hall and Dorsch, 1984). This initial step involves processing and presentation of T-Ag on the MØ surface; a process not fully understood. Tp must also recognize the "self" antigens or HLA-DR antigens of the MØ. The quantity of HLA-DR expressed by the MØ is crucial for Tp recognition and subsequent
Table 1. Various HLA antigens, as of 1982, according to HLA Nomenclature Committee (Schwartz, 1982).

<table>
<thead>
<tr>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-C</th>
<th>HLA-D</th>
<th>HLA-DR</th>
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<tbody>
<tr>
<td>HLA-A1</td>
<td>HLA-Bw4</td>
<td>HLA-Bw42</td>
<td>HLA-Cw1</td>
<td>HLA-Dw1</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>HLA-B5</td>
<td>HLA-Bw44(12)</td>
<td>HLA-Cw2</td>
<td>HLA-Dw2</td>
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<td>HLA-A3</td>
<td>HLA-Bw6</td>
<td>HLA-Bw45(12)</td>
<td>HLA-Cw3</td>
<td>HLA-Dw3</td>
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<tr>
<td>HLA-A9</td>
<td>HLA-B7</td>
<td>HLA-Bw46</td>
<td>HLA-Cw4</td>
<td>HLA-Dw4</td>
</tr>
<tr>
<td>HLA-A10</td>
<td>HLA-B8</td>
<td>HLA-Bw47</td>
<td>HLA-Cw5</td>
<td>HLA-Dw5</td>
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<tr>
<td>HLA-11</td>
<td>HLA-B12</td>
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<tr>
<td>HLA-Aw19</td>
<td>HLA-B13</td>
<td>HLA-Bw49(w21)</td>
<td>HLA-Cw7</td>
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<tr>
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<td>HLA-B15</td>
<td>HLA-Bw51(15)</td>
<td></td>
<td>HLA-DN9</td>
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<td>HLA-A25(10)</td>
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<td>HLA-Bw52(5)</td>
<td></td>
<td>HLA-Dw10</td>
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<tr>
<td>HLA-A26(10)</td>
<td>HLA-B17</td>
<td>HLA-Bw53</td>
<td></td>
<td></td>
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<tr>
<td>HLA-A28</td>
<td>HLA-B18</td>
<td>HLA-Bw54(w22)</td>
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<td>HLA-Aw32</td>
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<td>HLA-Aw43</td>
<td>HLA-B40</td>
<td>HLA-Bw62(15)</td>
<td></td>
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<tr>
<td>HLA-Bw41</td>
<td></td>
<td>HLA-Bw63(15)</td>
<td></td>
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</tr>
</tbody>
</table>
Figure 1. Cellular Events in Allograft Rejection
activation. If the density of HLA-DR is insufficient, the antigen-presenting function is decreased (Stobo, 1982). A second accessory function of Mφ is release of monokines, soluble products which promote or inhibit cellular responses. The monokine Interleukin-1 (IL-1), also known as Lymphocyte Activating Factor (LAF), must be present as well as adequate HLA-DR and T-Ag presentation in order for activation of Tp to a helper T-cell (Th) to occur. Following activation, Th release lymphokines such as Interleukin-2 (IL-2). IL-2, also called T-cell Derived Growth Factor (TDGF), promotes further maturation and proliferation of Th to cytotoxic T-cells (Tc) by binding to receptors for IL-2. The appearance of these receptors is dependent on antigen presentation (Larrson et al, 1980). These various steps of the immune response to an allograft are summarized in Figure 1. Greater detail on lymphocyte activation will be given in a later section. (See Cyclosporine A-Mechanism of Action).
Polyunsaturated Fatty Acids and Prostaglandins in Transplantation

The protective effects of polyunsaturated fatty acids (PUFA) in prevention of allograft rejection were investigated in both animal and clinical studies. Mertin reported linoleic acid enhanced survival of tail skin grafts in mice (Mertin, 1974; Mertin, 1976). Uldell initially reported that supplementation of conventional azathioprine and prednisone immunosuppressive regime with linoleic acid, in the form of sunflower seed oil, increased graft survival in ten renal allograft recipients (Uldell et al., 1974). Because of these encouraging results, Uldell began a two year double blind study testing the effects of PUFA supplementation in the treatment of renal allograft recipients. Initially, PUFA supplementation prolonged graft survival and function. This effect was not noticeable after six months (McHugh et al., 1977).

The beneficial effects of PUFA were thought to be due to conversion of linoleic acid to arachidonic acid and subsequent metabolism to prostanoids (Mertin, 1976; McHugh et al., 1977). Addition of PGE₂ increased survival of mouse skin allografts (Anderson et al., 1976), rat
renal allografts (Strom et al., 1976) and cardiac hamster-to-rat xenographs (Kakita et al., 1975). An additional study with mouse skin allografts tested a long acting synthetic analogue of PGE$_2$, 16,16-dimethyl PGE$_2$ methyl ester (Di-M-PGE$_2$), as well as the effects of indomethacin (IM), an inhibitor of prostaglandin synthesis. Enhanced graft survival occurred with Di-M-PGE$_2$ and Di-M-PGE$_2$ plus IM; however, IM alone enhanced graft rejection. These studies suggested the immunosuppressive effectiveness of exogenous and endogenous prostaglandins (Anderson et al., 1977).

Effects of Polyunsaturated Fatty Acids, Prostaglandins and Lymphocyte Function

Induction of lymphocyte transformation by the mitogen, phytohaemagglutin (PHA), or the antigen, purified protein derivative of tuberculin (PPD), are two established in vitro methods of testing immunocompetency of lymphocytes (Naspitz and Richter, 1968; Coulsin and Chalmers, 1967). Using these systems, Mertin and Hughes investigated the effects of saturated fatty acids (SFA) and PUFA on lymphocyte transformation in stimulated and unstimulated cultures. Transformation was determined by $^3$H-thymidine uptake. SFA and PUFA both decreased transformation in stimulated cultures. In unstimulated cultures, only SFA decrease $^3$H-thymidine uptake. This indicated that PUFA specifically inhibited PHA and PPD induced transformation, whereas SFA non-specifically inhibited lymphocyte transformation. They suggested the effect of PUFA may be mediated through prostanoids (Mertin and Hughes, 1975).
Initial studies found exogenous prostanoids or a synthetic prostaglandin analogue inhibited transformation and function of human lymphocytes stimulated by PHA (Lomnitzer et al., 1976; Smith et al., 1971; Mihas et al., 1975). Concentrations of prostaglandins used in these studies were significantly higher than concentrations of PGE produced by PHA stimulated leukocytes (approximately $1 \times 10^{-8}$M) (Ferraris and De-rubertis, 1974). Therefore, Goodwin et al., tested the ability of PGE$_1$ and PGE$_2$ at more physiological concentrations, to inhibit PHA-induced transformation. Three important observations were made in this study. First, 50 percent inhibition occurred at only $10^{-7}$M PGE. Also, addition of IM alone, actually enhanced lymphocyte transformation, due to the blockage of endogenous prostaglandin synthesis. In the presence of IM, only $10^{-8}$M PGE was needed to obtain 50 percent inhibition of transformation. Finally, IM enhancement was abolished if glass-adherent cells were removed from the lymphocyte preparation. This indicated that removal of these cells effectively removed endogenous PGE. Glass adherent cells were monocytes, B-cells and possibly glass adherent T-cells (Goodwin et al., 1977).

The previous studies correlated increased lymphocyte transformation with increased $^3$H-thymidine incorporation. Bockman and Rothschild studied PGE effects using another in vitro system, T-cell colony formation in soft agar cultures. This system provided visual evidence of T-cell proliferation induced by PHA. Exogenous PGE$_1$ and PGE$_2$, inhibited colony formation dose dependently with 50 percent inhibition occurring at $1.8 \times 10^{-7}$M. PGD$_2$ and PGF$_{2\alpha}$ had no effect. Addition
of IM enhanced colony formation. Additionally they found, in the presence of MØ, inhibition of T-colony formation consistently occurred. Concurrent addition of IM and MØ restored clonal growth (Bockman and Rothschild, 1979).

The mechanism of action of prostaglandin modulation of T-cell mitogenesis has not been established. This effect has been related to increased cAMP levels. However, this theory is still controversial (Goodwin and Webb, 1980). Studies have shown PGE inhibits Interleukin 2 (IL-2) production. IL-2 as previously mentioned, is a vital component of T-cell proliferation and differentiation. PGE$_1$ and PGE$_2$, but not PGF$_1$, PGF$_2$, PGA$_1$ or PGA$_2$, inhibit IL-2 production by T-cells. Depletion of MØ from T-cell cultures or addition of IM reduced this inhibition and IL-2 production increased (Rappaport and Dodge, 1982). A subsequent report found similar results, however the absolute removal of MØ from the T-cell cultures eliminated IL-2 production. This effect was attributed to removal of IL-1, the monokine that is essential for synthesis and release of IL-2 by T-cells. Thus, MØ may have the ability to modulate IL-2 release and subsequent T-cell proliferation by releasing IL-1 (positive control) and PGE (negative control) (Chouaib and Fradelizi, 1982).

**Immunosuppressants**

Adequate immunosuppression is the key element in successful organ transplantation. An ideal immunosuppressant should act specifically on events involved with the graft rejection and yet have no effect on other
immune responses needed for protection from disease. Historically, immuno­
suppressants have shown very little specificity.

Azathioprine (AZA), a purine analogue, was introduced by Calne in 1961 for use in renal transplantation (Calne, 1961). AZA inhibits DNA replication and subsequent cell proliferation of immunocompetent cells (Webb and Winkerstein, 1982). Unfortunately, AZA also inhibits mitosis of any replicating cell. Also, AZA depresses all immune function, allowing for potential infection.

Starzl et al. combined the use of steroids with AZA treatment in 1963 (Starzl et al. 1963). Steroids, such as prednisone (P), function by inhibiting; T-cell migration to sites of antigen, lymphokine mediated interactions between MØ and T-cells, and MØ microbicidal activity (Webb and Winkelstein, 1982; Borel, 1983). The combination of AZA plus P was the standard therapy for transplantation for many years. Side effects of this regime included; bone marrow depression, infections, pancreatitis, gastrointestinal hemorrhage, development of Cushingoid features, bone necrosis, cataract formation, vascular degeneration and cancer (Sheil et al, 1983).

Antilymphocyte serum (ALS) is another lymphocytotoxic therapy that is directed against immunocompetent cells. ALS, prepared by immunizing a heterologous species with cells from blood, spleen, thymus or lymph nodes, contains antibodies directed against the antigens present on lymphocytes. These antibodies bind to the HLA antigens and interfere with the response of the lymphocytes to these antigens. ALS treatment is complicated by unpredictable dosages needed to adequately prevent
rejection as well as serum sickness and nephritis due to repeated administration of a foreign heterologous serum (Webb and Winkelstein, 1982).

Cyclosporine A (CsA), the newest immunosuppressant, received a great deal of attention initially because it appeared to act specifically on T-cell mediated events. Recent advancements in transplantation have been attributed to the introduction and development of this compound (Kahan, 1983; Borel, 1983; Starzl, 1983). Extensive discussion of CsA is presented in a subsequent chapter (See Cyclosporine A).

MONOCYTE/MACROPHAGE (MØ)

Background

In 1891, Metchnikoff, a Russian zoologist, first discovered phagocytic cells in animal tissues; he called these cells "macrophages" (Werb, 1982; Akerman and Douglas, 1980). This simple observation initiated the isolation and characterization of a variety of cells currently classified as the Mononuclear Phagocytic Cells (MPC) (van Furth et al., 1975). In the bone marrow, MPC originates as monoblasts and develop into promonocytes, the precursors of monocytes. Monocytes leave the bone marrow and circulate throughout the body in peripheral blood. Monocytes leave the circulation, enter the tissue and mature into macrophages. Macrophages are found in many tissues such as lung (alveolar), liver (Kupffer), spleen, lymph nodes, and peritoneum (Akerman and Douglas; 1980; Douglas, 1982; Drutz and Mills, 1982; Werb, 1982).
Monocytes and macrophages do differ in location and morphology; however, they share certain common characteristics and functions; phagocytosis, secretion of soluble mediators of the immune response, interaction with antigen and lymphocytes in the generation of an immune response, expression of MHC-antigens and adherence to glass or plastic (Ackerman and Douglas, 1980; Douglas, 1982; Drutz and Mills, 1982; Werb, 1982). These functions are vital for the monocyte/macrophage (MØ) to perform as an immunocompetent cell.

Accessory Functions of MØ

As important as the effector phagocytic function of the MØ, are the accessory functions by which MØ modulate immunoresponsiveness of lymphocytes. These functions include secretion of biologically active products, monokines, and expression of cell surface antigens, HLA-DR antigens (Unanue, 1981).

One of the earliest monokines discovered was a protein factor, released from adherent peripheral blood cells, which enhanced the immune response of murine thymocytes to PHA. This factor was called Lymphocyte Activating Factor (LAF) (Gery et al., 1972; Gery and Waksman, 1972). Studies in rat and mouse systems showed MØ conditioned media promoted thymocyte and T-cell mitogenesis (reviewed by Unanue, 1981). LAF was renamed Interleukin-1 (IL-1) (Maizel and Ferrar, 1979) and methods for isolation and purification were developed (Lachman et al., 1980). Using purified human cells and IL-1, Maizel et al. confirmed results from earlier animal models. IL-1 augmented antigen-stimulated mitogenesis of
both human thymocytes and T-cells (Maizel et al., 1981). Greater detail
of the role of IL-1 in T-cell mitogenesis is discussed in other portions
of this dissertation.

In contrast to IL-1, prostaglandins released from MØ are
considered down regulators of the immune response (Kurland and Bockman,
1978; Unanue, 1980; Kennedy et al., 1980, Goldyne and Stobo, 1981,
Goodwin et al., 1977). The effects of prostaglandins on lymphocyte
transformation were discussed previously (see Polyunsaturated Fatty
Acids and Prostanoids in Lymphocyte Transformation).

Early studies suggested that the MØ was the source of inhibitory
prostaglandins since removal of adherent cells effectively reversed
inhibition of lymphocyte transformation (Goodwin et al., 1977, Bockman
and Rothschild, 1979; Goodwin and Webb, 1980). Though the ability of
lymphocytes to produce prostaglandins was reported (reviewed by Goldyne
and Stobo, 1981), MØ contamination of lymphocytes was not adequately
prevented. Studies with highly purified lymphocytes reported no
detectable PGE2 levels following stimulation by PHA and other lectins
(Kurland and Bockman, 1978; Yamamoto et al., 1979; Kennedy et al.,
1980). Perhaps the most conclusive study to determine if lymphocytes or
MØ were the source of prostanoids was conducted by Kennedy et al.
Highly purified T-cells and MØ were labeled with 14C-20:4(n-6).
MØ synthesized both 14C-TXB2 and 14C-PGE2; T-cells stimulated
by PHA failed to synthesize any 14C-prostanoids however,
14C-20:4(n-6) was released. Addition of unlabeled MØ to stimulated
14C-T-cells resulted in increased 14C-PGE2 and 14C-TXB2
production. Thus, PHA stimulated release of $^{14}$C-20:4(n-6) from T-cells which was utilized by MØ and converted to prostaglandins (Kennedy et al., 1980).

A variety of prostanoids have been isolated from MØ. Prostanoids isolated from murine MØ included: PGE$_2$, PGF$_{2\alpha}$, PGI$_2$, PGD$_2$ and TXA$_2$ (peritoneal); PGE$_2$ (spleen); PGE$_2$, TXA$_2$ and PGI$_2$ (bone marrow). MØ from other species produce similar products: PGE$_2$, PGF$_2$, and PGI$_2$ (rat peritoneal); PGE$_2$, TXA$_2$ and PGI$_2$ (guinea pig peritoneal) and PGE$_2$, PGF$_{2\alpha}$, PGI and PGD$_2$ (rabbit alveolar) (reviewed by Goldyne and Stobo, 1981). The major prostanoids isolated from human peripheral blood MØ included PGE$_2$, PGI, and TXA$_2$ (Kurland and Bockman, 1978; Kennedy et al., 1980; Goldyne and Stobo, 1981; Pawlowki et al., 1983).

The Role of MØ in Atherosclerosis

The development of the characteristic lesions of atherosclerosis; the fatty streak, fibrous plaque and complicated lesion, involves the following processes: internal smooth muscle cell proliferation; formation of connective tissue matrix by smooth muscle; and development of foam cells, lipid laden smooth muscle and MØ. (Ross, 1979a; 1979b; 1981; Schwartz and Ross, 1984). A number of cell types; endothelial, smooth muscle, MØ and platelets, have been implicated as active participants in the initiation and propagation of this disease through the release of growth factors (Figure 2.). One model, the Injury Response Hypothesis, postulates that initiation of atherosclerosis
Figure 2. Growth Factors Released by Various Cell Types (Ross, 1981)
begins with injury to the endothelial lining of the artery. MØ and platelets respond to injury, attach to the lining and release soluble growth factors which promote smooth muscle cell proliferation. If injury is limited, the endothelial lining is reconstituted. However, if injury is prolonged, lesion formation progresses (Ross, 1979a; 1979b; 1981; Schwartz and Ross, 1984).

An experimental model designed to study the progression of the atherosclerotic lesion is diet-induced hypercholesterolemia in various animals. A recent study using non-human primates showed elegant morphological evidence of the cells which participate in changes that lead to fatty streak formation (Faggiotto et al., 1984) and fatty streak conversion to fibrous plaque (Faggiotto and Ross, 1984). Electron microscopic analysis of various segments of the aortic arch, thoracic aorta and abdominal aorta showed MØ adhered to endothelium within 12 days of cholesterol feeding. The MØ migrated below the endothelial lining and accumulated lipid. Within two months, subendothelial foam cells had formed fatty streaks consisting of lipid laden MØ and smooth muscle cells. By four months, the endothelial lining was disrupted and subendothelial foam cells were released into peripheral blood and exposed macrophages were covered by platelets. This study concluded that the most obvious initial changes in the artery were associated with MØ-endothelial interactions (Faggiotto et al., 1984).

The MØ is involved in two major aspects of developing atherosclerosis; the accumulation of lipid in foam cell formation and promotion of smooth muscle cell proliferation by MØ derived growth
factors. MØ, and to a lesser extent, SMC develop into foam cells (Ross, 1981; 1984; Gerrity, 1981; Chait and Mazzone, 1982; Mazzone and Chait, 1982; Paites et al., 1983; Brown and Goldstein, 1983; Steinberg, 1983; Faggiotto and Ross, 1984). This process is not yet fully understood, however studies have shown MØ are capable of lipid accumulation by receptor mediated uptake of modified low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (Shechter et al., 1981; Mazzone and Chait, 1982; Paites et al., 1983; Brown and Goldstein, 1983; 1984; Steinburg, 1983).

Because increased smooth muscle cell proliferation is a key element of developing lesion, release of mitogenic factor from MØ would be a proatherosclerotic element. A vital component of normal wound repair is a mitogenic factor produced by infiltrating MØ. Elimination of MØ effectively prevents healing (Leibovich and Ross, 1975; Hunt and Van Winkle, 1976). Numerous studies reported a MØ derived growth factor, derived from a variety of tissue MØ, stimulated growth of fibroblast, smooth muscle and endothelial cells (Leibovich and Ross, 1976; Unanue and Kiely, 1977; Wall et al., 1978; Greenburg and Hunt, 1978; Goldman and Bar-Shavit, 1979; Jalkanen et al., 1979; Wahl et al., 1979; Martin et al., 1980). Studies with human peripheral blood monocytes also demonstrate the presence of MØDGF. Glenn and Ross found MØDGF was not immediately produced by peripheral MØ but did occur after MØ were placed in culture for four to five days. They suggested differentiation of peripheral blood MØ to a macrophage type cell occurred. Thus, peripheral blood MØ did not have the capacity to
produce MØDGF. MØDGF release was stimulated by endotoxin and Concanavalin (ConA) (Glenn and Ross, 1981). In contrast, another study using peripheral blood MØ, found, following 18 hour incubation, MØ-conditioned media, MØM, stimulated growth of SMC cultures maintained for a period of six days. By Day 3 there were significant changes in SMC growth which by Day 6 were extremely evident. Stimulation of MØ with zymosin, opsonized zymosin and latex particles enhanced the growth promoting ability of MØM (Ziats, and Robertson, 1981).

Unlike platelet derived growth factor (PDGF), MØDGF has not been well characterized. Some reports suggest IL-1 is responsible for the mitogenic effects of MØM (Schmidt et al., 1982). However, recent studies have shown MØDGF and IL-1 are "distinct entities" (Bitterman et al., 1982; Estes, et al., 1984).

CYCLOSPORINE A

Structure

Cyclosporine A (CsA) was initially isolated from the fungal species Tolypocladium inflatum Gams (Dreyfuss et al., 1976; Ruegger et al., 1976). The structure of CsA was elucidated by chemical degradation and analysis of an iododerivative by x-ray crystallography (Ruegger et al., 1976; Petcher et al., 1976). CsA is a neutral, hydrophobic, cyclic endecapeptide with a molecular weight of 1202.6, that is readily dissolved in ethanol (Figure 3).
Natural analogues of CsA exist which share commonly the cyclic endecapeptide structure. Variations occur only as a single amino acid substitution within the basic structure of the compound. These analogues are Cyclosporine B ([Ala\textsuperscript{2}] cyclosporine), Cyclosporine C ([Thr\textsuperscript{2}] cyclosporine), Cyclosporine D ([Val\textsuperscript{2}] cyclosporine) and Cyclosporine E ([Val\textsuperscript{11}] cyclosporine).

Wenger, has described the chemical synthesis of CsA and various synthetic analogues. The relative biological potencies of the synthetic
and naturally occurring analogues were compared to determine the relationship between structure and activity (Table 2). The preliminary conclusions reached were:

1) Modification of amino acid 1 such as; the removal of the nonpolar side chain ([MeThr'] cyclosporine), modification of the hydroxy group ([O-acetyl] and [Desoxy] cyclosporine) or reduction of the double bond ([Dihydro] cyclosporine) reduced biological activity (Wenger, 1983).

2) Substitutions at amino acid 2 were not as critical if the hydrophobicity was not altered. Decreased activity occurred when serine replaced the α-aminobutyric acid of CsA or the threonine of CsB (Wenger, 1983).

3) Conformational changes also altered biological effectiveness. The substitution of D-MeVal for L-MeVal at amino acid 11, significantly reduced activity. Additionally, D-Pro as amino acid 3 resulted in decreased potency indicating that though conformation was not drastically altered, some steric hindrance occurred and prevented efficient binding to a possible receptor (Wenger, 1983).

Experimental and Clinical Applications

In 1976, Borel et al. initially reported the anti-lymphocytic action of CsA. They found CsA was immunosuppressive in model systems of humoral immunity (reduction of plaque forming cells and haemagglutinin titres in mice) and cell mediated immunity (skin graft rejection, graft vs. host disease, and experimental encephalomyelitis) (Borel et al., 1976). A subsequent article reported CsA directly inhibited T-cells yet had little effect on B-cell mediated events. Additionally, little cellular destruction of haemopoietic tissues occurred with high doses of CsA (Borel et al., 1977). These initial studies suggested that CsA might prove to be an ideal immunosuppressant to prevent rejection in organ transplantation. The unique anti-lymphocytic specificity as well
Table 2. Biological Activity of Various Natural and Synthetic Cyclosporine Analogue (Wenger, 1983)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity</th>
<th>Source</th>
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<tbody>
<tr>
<td>Cyclosporine</td>
<td>***</td>
<td>Natural</td>
</tr>
<tr>
<td>(MeThr&lt;sup&gt;1&lt;/sup&gt;) cyclosporine</td>
<td>*</td>
<td>Synthetic</td>
</tr>
<tr>
<td>(Thr&lt;sup&gt;2&lt;/sup&gt;) cyclosporine</td>
<td>***</td>
<td>Natural</td>
</tr>
<tr>
<td>(Ser&lt;sup&gt;2&lt;/sup&gt;) cyclosporine</td>
<td>**</td>
<td>Synthetic</td>
</tr>
<tr>
<td>(Ala&lt;sup&gt;2&lt;/sup&gt;) cyclosporine</td>
<td>**</td>
<td>Natural</td>
</tr>
<tr>
<td>(Val&lt;sup&gt;2&lt;/sup&gt;) cyclosporine</td>
<td>**</td>
<td>Natural</td>
</tr>
<tr>
<td>(nVal&lt;sup&gt;2&lt;/sup&gt;) cyclosporine</td>
<td>***</td>
<td>Natural</td>
</tr>
<tr>
<td>(D-Pro&lt;sup&gt;3&lt;/sup&gt;) cyclosporine</td>
<td>*</td>
<td>Synthetic</td>
</tr>
<tr>
<td>(D-MeVal&lt;sup&gt;11&lt;/sup&gt;) cyclosporine</td>
<td>*</td>
<td>Natural/Synthetic</td>
</tr>
<tr>
<td>(MeLeu&lt;sup&gt;11&lt;/sup&gt;) cyclosporine</td>
<td>*</td>
<td>Synthetic</td>
</tr>
<tr>
<td>(O-acetyl)-cyclosporine</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>3'-Desoxy-cyclosporine</td>
<td>*</td>
<td>Natural</td>
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<tr>
<td>Dihydro-cyclosporine</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>H-MeBmt-OH</td>
<td>*</td>
<td>Synthetic</td>
</tr>
</tbody>
</table>

*** Indicates potent immunosuppressant activity  
** Intermediate activity  
* Little or no activity
as low myotoxicity of CsA were definite advantages over immunosuppressive methods used previously.

In a comprehensive review, White and Calne detailed the progression of CsA use in organ transplantation from initial trials in animals to successful clinical applications in Man (White and Calne, 1981). Kostakis et al. first tested CsA in a rat heterotopic heart transplant model. The initial experiments failed due to improper administration, intermuscular injection of CsA in ethanol, and excessive doses of CsA (Kostakis et al., 1977a). Subsequent experiments with lower amounts and administration of the drug in olive oil, resulted in prolonged graft survival (Kostakis et al., 1977b). Next, CsA was tested in two different animal models; kidney allografts of mongrel dogs (Calne and White, 1977) and heart grafts of pigs (Calne and White, 1978a). In both cases, CsA successfully prevented rejection better than any other immunosuppressive regime. These studies effectively demonstrated the ability of CsA to deter rejection of allografts not only among highly inbred animals, rats, but also a more difficult case, among animals with very little genetic homogeneity.

Following these initial studies, CsA was tested with a variety of organ transplants among a variety of species (Table 3). CsA successfully prevented allograft rejection of: the kidney in rat, rabbit, dog and monkey; skin in rat, mouse, rabbit and dog; the heart in rat, pig and monkey and the liver in rat. In many instances, rejection occurred shortly after CsA administration was stopped (S). However, prolonged graft survival occurred in rat kidney, heart and bone marrow

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Rat</th>
<th>Mouse</th>
<th>Rabbit</th>
<th>Dog</th>
<th>Pig</th>
<th>Monkey</th>
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<tr>
<td>KIDNEY</td>
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<td>BONE MARROW</td>
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<td>PANCREAS/ISLET</td>
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<td>HEART-LUNG</td>
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</table>
transplants as well as pig heart allografts, despite cessation of CsA (S*). These studies suggested the exciting possibility that CsA had the potential to induce graft acceptability; thus, negating the need for continued drug-induced immunosuppression (Calne and White, 1981).

Additionally, CsA enabled successful transplantation of organs not previously attempted such as lung in dog, lung and heart in monkey, corneas in rabbit, or pancreas in dog. CsA prolonged survival of pancreatic islets transplanted as a vascularized composite graft of kidney placed under the renal capsule of diabetic rats, however CsA was ineffective with isolated islet transplantation in the rat and dog. Additionally, CsA enabled successful bone marrow transplantation across minor histocompatibility barriers in rat and mouse. However, CsA did not effectively prevent bone marrow rejection of non-histocompatible dogs (Borel, 1983).

Calne et al. conducted the first clinical trials of CsA in patients receiving cadaveric organ transplants (32 kidneys, two livers and two pancreata) (Calne et al., 1978b, 1979). The results of this initial pilot study found CsA could act as an effective immunosuppressant, 26 kidneys, both livers and pancreata were functioning after one year. It was necessary to supplement CsA treatment with steroids in some cases, however, 15 kidneys never received additional immunosuppressants. Minor side effects of CsA included; increased hair on face and body (hirsutism), gum hypertrophy, and slight tremor. Of major concern were two additional adverse effects; nephrotoxicity and, in three cases, appearance of lymphomas. They concluded that CsA showed promise as an
immunosuppressant because it was steroid sparing, non-myelotoxic and easily administered. However, they cautioned that the harmful side effects, nephrotoxicity and lymphoma, warranted extreme care in future clinical efforts (Calne et al., 1979).

Starzl et al. began the first American clinical trial of CsA in 22 renal transplant recipients (Starzl et al., 1980). Their results were strikingly more optimistic. They found that initial rejection episodes were reversed if CsA treatment was supplemented by low doses of prednisone. Of the original 22 patients, 19 had functional grafts, two patients rejected their grafts and one patient died of surgical complications. The side effects observed were similar to those seen by Calne with the exception of lymphoma; no tumors appeared in any patients. Nephrotoxicity occurred in seven patients but renal function improved with decreased CsA dosage. They concluded CsA plus prednisone (CsA-P) therapy was superior to conventional therapy since CsA allowed for a lowered dose of steroid thus reducing the detrimental side effects associated with steroid immunosuppression (Starzl et al., 1980).

The initial trials by Calne and Starzl initiated an avalanche of research testing the efficacy of the promising new immunosuppressant. Summaries of a number of studies concerning the use of CsA in kidney, heart, liver, pancreas and bone marrow were presented at the First International Congress of Cyclosporine in May of 1983.

The ability of CsA to prevent rejection of renal transplants in man has been extensively examined. The majority of studies found CsA either alone (Sheil et al., 1983; Sells et al., 1983) or supplemented with low
doses of prednisone (Starzl et al., 1983a; Kahan et al., 1983; Land et al., 1983, McMaster et al., 1983 and Buzendahl et al., 1983) was more effective than conventional azathioprine and prednisone (AZA-P) therapy. Two studies compared CsA-P to azathioprine plus prednisone plus antilymphocyte globulin (AZA+P+ALG). The studies reported both treatments were equally effective and suggested AZA-P-ALG was preferable due to decreased incidence of nephrotoxicity (Najarian et al., 1983, and Halloran et al., 1983). The use of conventional immunosuppressants often causes severe and sometimes fatal complications in certain categories of people. Large doses of steroids or the use of AZA cause detrimental side effects in children, the elderly, diabetics and AZA sensitive individuals. One study reported among the elderly (over age 55) graft and patient survival of 73 and 85 percent, respectively, with CsA-P treatment in comparison to 41 and 59 percent with AZA-P treatment. In AZA sensitive patients 100 percent graft survival occurred with CsA-P and, finally with diabetic patients, 75 percent survival occurred 3 - 37 months after transplantation (Ringden et al., 1983). CsA was also found to be an effective alternative in children (Conley et al., 1983).

The majority of CsA studies investigated the effectiveness of CsA in cadaveric kidney transplantation. This situation presents the largest histocompatibility difference and thus the greatest immunosuppressive challenge. However, in the case of renal transplantation, often an organ is available from a living relative. A study with parent to child transplants found CsA treatment to be superior to conventional AZA-P therapy with no graft rejection at time of report (Ota et al., 1983).
In 1980, CsA was first used in cardiac transplantations at Stanford (Oyer et al., 1983). Initial success prompted continued use of this agent in all subsequent operations. A summary of results two-and-a-half years after the initial trials found improved survival rates of patients with CsA therapy. Recuperation rates improved due to the reduction of complications that often resulted with large amounts of steroids. Some CsA side effects occurred; decreased renal function possibly due to nephrotoxicity, increased hypertension, lymphomas and accelerated allograft coronary atherosclerosis in a few patients (Oyer et al., 1983). Other studies found greater success with CsA+P supplemented with ALG. The addition of ALG reduced initial episodes of rejection (Hardesty et al., 1983 and Wallwork et al., 1983).

Success of liver transplantation has dramatically increased since the introduction of CsA to this procedure in 1980 (Calne et al., 1981; Starzl et al., 1981). Reports show that CsA+P is the most effective immunosuppressive regime available (Starzl et al., 1983b; Wonigeit et al., 1983). One difficulty encountered with CsA was initial malabsorption. This was possibly due to either inadequate emulsification of the drug because of disturbed bile secretion or interference in enterhepatic recirculation (Wonigeit et al., 1983). This agent was not effective in preventing pancreatic transplant rejection in Man (Sutherland, 1983; Traeger et al., 1983; Sutherland et al., 1983; and Traeger et al., 1984). Also, only slight success was achieved with CsA in bone marrow transplantation (Tutscka et al., 1983; Speck et al., 1983 and Storb et al., 1983).
The side effects of CsA therapy include tremors, hirsutism (growth of excess facial and body hair), gingival hyperplasia (excess growth of gum tissue), hypertension (in cardiac transplantation), lymphoma and nephrotoxicity (Starzl, 1983; Ota and Bradley, 1983). The initial three complications are successfully reduced with decreased CsA dosage. Hypertension occurs in the majority of cardiac transplant recipients treated with CsA-P as opposed to AZA-P. The appearance of lymphomas has greatly decreased with greater understanding of proper CsA dose and the tendency towards over-immunosuppression. Epstein-Barr virus (EBV) infection has been associated with the development of post transplant lymphomas (Hanto et al., 1981; Penn, 1983). Latent EBV-infected B-cells are normally suppressed by viral specific cytotoxic-T-cells. The specific action of CsA on T-cell proliferation may disrupt this delicate EBV surveillance system (Britton and Palacios, 1982; Dhein et al., 1983). Nephrotoxicity is perhaps the most detrimental side effect of CsA resulting in decreased renal function in the majority of transplant recipients. This toxicity mainly involves proximal tubular lesions and is indicated by increased serum creatine levels. Changes appear reversible in some instances by adjusting serum levels (Ota and Bradley, 1983).

**Mechanism of Action**

In order to understand the postulated mechanism of action of CsA, some understanding of the process of T-cell activation is needed. T-cell activation has been described by a 3-step sequence of events
(Lafferty et al., 1980; Andrus and Lafferty, 1982; Lafferty et al., 1983).

\[ T \rightarrow^T T' \]  

(1)

\[ T \rightarrow^T T', IL-2 \]  

(2)

\[ T' \rightarrow^T IL-2 \]  

(1)

\[ IL-2 \rightarrow^T nT' \]  

(2)

\[ T' \rightarrow^T T', nT' \]  

\[ T \rightarrow^T T', IL-2 \]  

\[ IL-2 \rightarrow^T nT' \]  

(T) represents the resting precursor T-cell, (T') is the activated T-cell, signal (1) represents antigen binding to the T-cell, signal (2) is the monokine IL-1 and (nT') represents clonal expansion of activated T-cells. Two components are necessary for initial activation, antigen presentation by accessory cells such as MØ and co-stimulation by IL-1 (reaction a). Activated T-cells express IL-2 receptors and effector function. Antigen binding will in turn stimulate synthesis and release of IL-2 and other lymphokines (reaction b). IL-2, originally named T-cell Growth Factor, promotes clonal growth and expansion of activated T-cells (reaction c). This process explains activation and expansion of all T-cell subsets (helper, suppressor, and cytotoxic). Additionally, each subset can express cytotoxic activity (Juretic et al., 1981; Lafferty et al., 1983).
Though the exact mechanism of action of CsA has not been determined, it is accepted that CsA inhibits early stages of T-cell activation (Britton and Palacios, 1982; Andrus and Lafferty, 1982; Hess et al., 1983; and Lafferty et al., 1983). Clonal expansion, reaction c, of activated T-cells is not inhibited by CsA (Bunjes et al., 1981; Dos Reis and Shevachk, 1982; Orosz et al., 1982; Andrus and Lafferty, 1982). Lymphokine release from activated T-cells, reaction b, is inhibited in the presence of CsA (Orosz et al., 1982; Andrus and Lafferty, 1982). Antigen binding, a necessary component of this process does not appear to be prevented, thus, CsA may inhibit biochemical events following antigen binding (Lafferty et al., 1983). CsA also inhibits the primary T-cell activation step, reaction a (Larsson and Coutinho, 1979; Andrus and Lafferty, 1982). Again, antigen binding is vital as well as co-stimulation by IL-1. CsA inhibits IL-1 production by MØ (Bunjes et al., 1981; Tosato et al., 1982).

One aspect of the mechanism of action of CsA includes the route by which CsA contacts or enters the cell. The presence of a membrane bound receptor has been suggested (Ryffel et al., 1980; 1982). Additional studies questioned these findings. Le Grue et al. compared $^3$H-CsA uptake in lymphocytes and kidney cells and found "binding constants" based on Scatchard analysis to be similar. This led them to measure uptake and release of CsA by liposomes containing either phosphatidylcholine and cholesterol alone or supplemented with lipids extracted from normal peripheral blood lymphocytes. They found no differences in these parameters between the liposome and lymphocytes. They concluded
that hydrophobic CsA partitions into the lipid phase of the membrane unassisted by a protein receptor (Le Grue et al., 1983a; 1983b).

Unlike the membrane bound receptor, a cytosolic binding protein for CsA has been isolated. The presence of this receptor was initially found in BW 5147-cells, a malignant T-cell line (Merker et al. 1983). "Cyclophilin" has been subsequently isolated from calf thymus and characterized as a protein with a molecular weight of 15,000. The affinity of this protein for both natural and synthetic analogues of CsA correspond nicely to their immunosuppressive efficiency. Cyclophilin activity has been detected in cytosol extracts of murine thymus, mature T-cells, kidney and brain as well as human thymus and mature T-cells. The existence of this protein in the cytosol indicates an intracellular mechanism for the immunosuppressive activity of CsA (Handschumacher et al., 1984).
MATERIALS AND METHODS

MATERIALS

Arachidonic acid [20:4(n-6)] (NuChek, Elysian, MN) was shown to be peroxide free by thin layer chromatography (Huttner, 1977b). Medium for growing smooth muscle cells to confluency (growth medium) was prepared from 1X Eagle's minimum essential medium which contains Hank's salts and 25 mM HEPES buffer (GIBCO, Grand Island, NY) supplemented with 50 μg/ml gentamycin sulfate (Schering, Kenilworth, NJ), 2 mM glutamine, 1X nonessential amino acids (Microbiological Associates, Walkersville, MD), 1mM pyruvate and 1-3 mg/ml of sodium bicarbonate. The medium was supplemented with 10 percent fetal bovine serum (FBS) (Sterile Systems, Logan, VT; Hyclone lots 100348 and 010439). Antiserum for the radioimmunoassay (RIA) of PGI$_2$ (measured as 6-keto PGF$_{1\alpha}$) and PGE$_2$ were kindly supplied by Dr. L. Levine, Brandeis University. The cross reactivity of PGE$_2$ antibody was: PGE$_2$, 100%; PGE$_1$, 70%; 6-keto-PGF$_{1\alpha}$, 0.4%; PGF$_1$, 0.76%; PGF$_2$, 0.31%; PGD$_2$, 0.051%; 20:4(n-6), 0.00045%, and CsA, 0.0%. The cross reactivity of 6-keto-PGF$_{1\alpha}$, 0.15%; PGD$_2$, 0.02%, PGF$_2$, 0.10%, 20:4(n-6), 0.005% and CsA, 0.0%. Indomethacin was purchased from Sigma Chemical Company, (St. Louis, MO), and stock solution diluted in RPMI 1640 (Microbiological Associates, Walkersville, MD), for addition to MØ cultures or in 100 percent ethanol in SMC growth studies.
PREPARATION OF HUMAN MØ

Peripheral blood mononuclear cells (PBMC) were obtained by the centrifugation of blood from normal donors over Ficoll-Hypaque gradients (Boyum, 1976). The PBMC were rinsed twice in Seligmanns balanced salt solution (SBSS) (GIBCO, Grand Island, NY) containing 2% human albumin and resuspended at 10 x 10^6 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Microbiological Associates, Walkersville, MD). Five ml of the cell suspension were added to each 100 mm diameter plastic petri dish (No. 25020, Corning Glassworks, Corning, NY). Following an 18-hour or 1-hour incubation at 37°C in 5% CO2, 95% air, the nonadherent cells were removed by rinsing the petris with 37°C SBSS-2% albumin. The adherent MØ were gently harvested with a rubber policeman and 90% or more of the cells routinely demonstrated esterase activity (Yam, 1971).

CULTURING OF HUMAN MØ

The MØ were adjusted to 1 x 10^6/ml in RPMI-10% FBS and 2 ml cultures performed in multiwell tissue culture plates (No. 3047, Falcon Plastics, Oxnard, CA). The cultures were incubated at 37°C in 5% CO2, 95% air. After specified incubation times, the medium was removed and centrifuged at 1500 x g. The supernatants were harvested for the PGE2 determinations.
RADIOIMMUNOASSAY FOR PGE$_2$ AND 6-KETO-PGF$_{1\alpha}$

The PGE$_2$ and 6-keto-PGF$_{1\alpha}$ concentrations in culture supernatants were determined with the following radioimmunosassay procedure. Culture supernatants were diluted in Tris-albumin buffer (pH 7.5). Each sample was incubated for 18 hours at 4°C with a predetermined optimal dilution of rabbit antiserum directed towards PGE$_2$ or 6-keto-PGF$_{1\alpha}$ and with $^3$H-PGE$_2$ (Sp. Act. 438 mCi/mg, Cat. No. TRK. 431, Amersham, Arlington Heights, IL) or $^3$H-6-keto-PGF$_{1\alpha}$ in a total final volume of 600 ul. Each sample then received 500 ul of a 0.05% dextran/0.5% charcoal suspension in Tris-albumin buffer to absorb the free prostaglandin. The samples were immediately centrifuged at 3000 g for 20 minutes at 4°C and 200 ul of the supernatants removed. The supernatants were brought up to 6 ml in ACS II scintillation fluid (Amersham) and the radioactivity measured with a Beckman LS 8100 counter. Standard curves were constructed by plotting the radioactivity obtained in assays performed with known concentrations of prostaglandin. The PGE$_2$ and 6-keto-PGF$_{1\alpha}$ concentrations present in the culture supernatants were calculated from the standard curve obtained for each series of experiments.
QUANTITATION OF HLA-DR ON MØ BY FLOW MICROCYTOFLUOROMETRY (FMF) ANALYSIS

To determine the expression of HLA-DR antigens, the MØ were incubated at 3 x 10^6 cells/ml with a 1/50 final concentration of anti-HLA-DR monoclonal reagent (New England Nuclear, Boston, MA). The cells were incubated for 1 hour at 4°C and rinsed in Seligmann's balanced salt solution (SBSS) (Grand Island Biological Co., Grand Island, NY) supplemented with 0.1% sodium azide (NaN_3). A 1/10 dilution of fluorescinated F(ab')2 fragments from sheep antisera directed against mouse IgG (Cappel Laboratories, Cochranville, PA) was added to 3 x 10^6 cells suspended in 50 ul of SBSS. After incubating at 4°C for 30 minutes, the cells were rinsed and resuspended in SBSS plus NaN_3 for FMF on a No. 504 Orthocytofluorograf system. The cytofluorogram distribution profiles represented the relative fluorescence intensities of cells plotted on the X axis versus the number of cells on the Y axis.

QUANTITATION OF MØ CELLULAR VOLUME

Cellular volume was quantitated with a Coulter channelyzer (Curin Matheson Scientific, In., Houston, TX) as previously described (Cuthbert and Lipsky, 1981). The MØ suspensions were prepared, rinsed twice, and gently pipetted to obtain single cell suspensions immediately before channelyzer analysis. The numbers of cells in the 100 individual 10 um channels were measured and the volume distribution profiles plotted on an X-Y recorder.
PREPARATION OF MONOCYTE CONDITIONED MEDIA (MØM)

MØ were suspended in experimental media containing either 10% FBS (growth studies) or 2 x 10^6 cells/ml. MØ were incubated for 24 hours at 37°C, unless indicated. Media was removed from cultures and centrifuged at 600g to remove any MØ. MØM was either used fresh or after storage at -20°C. Storage did not effect MØM. MØM was made as described above with the addition of 10 uM IM prior to initial incubation.

TISSUE CULTURE OF SMOOTH MUSCLE CELLS

Primary cultures of smooth muscle cells (SMC) were established from prepubertal males as described (Huttner et al., 1977a). Cells were maintained in growth medium.

PROSTANOID SYNTHESIS IN CONFLUENT SMC CULTURES TREATED WITH CsA

Cells were used at passage level 3 and were seeded at 1.3 x 10^4 cells/cm^2 in Corning T-25 flasks containing 4 ml of experimental medium, growth medium supplemented with 1X essential vitamins, 1X essential amino acids and 20% FBS. The cells were allowed to grow to confluency
for four days. Media was removed and fresh media containing solutions of CsA and/or 20:4(n-6) in ethanol. Control cultures were treated with equal quantities of ethanol. The 6-keto PGF$_{1\alpha}$ (PGI$_2$) in media was measured after a 24 hour incubation period by RIA.

**PROSTANOID SYNTHESIS IN CONFLUENT SMC CULTURES TREATED WITH MØ or MØ-CONDITIONED MEDIA (MØM)**

SMC cultures were set up as previously described. At confluency, SMC cultures were treated with fresh media containing 3 mls of experimental media plus 1 ml of either 2 x $10^6$ MØ suspended in experimental media with or without 120 uM 20:4(n-6), or 1 ml of MØM with or without 120 uM 20:4(n-6). Following 24 hour incubation, media was removed and centrifuged to remove any contaminating cells and PGE$_2$ and 6-keto-PGF$_{1\alpha}$ levels were measured by RIA.

**SMC CELL PROLIFERATION WITH CsA**

Smooth muscle cells, passage 3 and 3-5 days post confluent, were seeded at low density (80 cells/cm$^2$) on tissue culture dishes (60 x 15 mm). Cells were allowed to attach to the plastic petri plates for one day and then treated with ethanol solutions of CsA and/or IM in fresh media containing 10% FBS. Control cultures were treated with equal quantities of ethanol. Cells were retreated with a media change on day 5 of the incubation period. After an 8-day incubation period, cells
were fixed with 3.7% phosphate buffered formalin and stained with filtered Giemsa. Prior to fixing, aliquots of media were removed for RIA analysis of prostanoid. Total cell area was measured by image analysis reported in arbitrary units (5361 units/cm²). A relative count was obtained from the total cell area on the plate. The relationship between cell area and cell number has been validated with a microscope and a Coulter counter (Gavino et al., 1982; Morisaki et al., 1982a; Morisaki et al., 1982b) cells from the same primary and the same batch of growth medium were compared in each treatment group.

SMC CELL PROLIFERATION WITH MÔM

SMC cultures were set up as described in the previous section. Cultures were treated with a solution of experimental media containing 20% MÔM or MÔMIM. IM was added in ethanol solution in appropriate cultures. Cultures were pretreated with identical solutions. Media was removed and prostanoid levels were measured by RIA. Cells were fixed, stained and counted as described in the previous sections. Results are given as relative growth.

STATISTICS

In order to determine the significance of difference between means, various statistical procedures were used. They include: analysis of variance, paired t-test and the Tukey-HSD test. The paired t-test and
the Tukey-HSD test, a multiple range procedure, yielded levels of significance. The particular procedures used are indicated in the table legends.
RESULTS

CsA AND PGI₂ BIOSYNTHESIS IN SMC

Confluent cultures of SMC were used to test the effect of CsA on PGI₂ biosynthesis. Cultures were treated with amounts of CsA ranging from 0.1 uM - 100 uM CsA. After a 24 hour incubation, the media was removed and levels of PGI₂ (measured as 6-keto-PGF₁α) were determined by RIA. CsA stimulated PGI₂ synthesis in a dose dependent manner reaching a maximum at 50 uM CsA. A decrease in PGI₂ occurred at 100 uM CsA. The presence of floating cells indicated CsA was cytotoxic at this concentration (Table 4).

Confluent cultures were used to test the effects of CsA, in the presence of exogenous 20:4(n-6), on PGI₂ biosynthesis. As expected, the addition of 20:4(n-6) enhances PGI₂ synthesis beyond control levels. There was no dose dependent stimulatory effect of CsA on PGI₂ release in the presence of exogenous 20:4(n-6) (Table 4). This indicated that CsA increases PGI₂ synthesis by stimulating endogenous release of substrate (20:4(n-6)) as opposed to stimulating conversion of 20:4(n-6) to PGI₂ (Cornwell and Morisaki, 1984; Morisaki et al., 1983).
Table 4. Effect of CsA on PGI$_2$ Synthesis
(6-keto-PGF$_{1\alpha}$) in SMC (Lindsey et al., 1983)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prostanoid (nmol/culture)</th>
</tr>
</thead>
</table>

1. Confluent Cells

<table>
<thead>
<tr>
<th>Media</th>
<th>Prostanoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>0.90±0.11</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.90±0.11</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.93±0.08</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>1.45±0.15</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>2.41±0.17</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>1.57±0.19</td>
</tr>
</tbody>
</table>

120 uM 20:4 (n-6) | 4.21±0.23 |
| " "  | 4.14±0.30 |
| " "  | 4.23±0.35 |
| " "  | 4.40±0.31 |
| " "  | 4.86±1.01 |
| " "  | 4.22±0.46 |

II. Proliferating Cells

<table>
<thead>
<tr>
<th>Media</th>
<th>Prostanoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>0.0215±0.0005</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.0227±0.0004</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.0232±0.0008</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.0253±0.0006</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.0269±0.0009</td>
</tr>
</tbody>
</table>
Table 5. Effect of CsA on the Proliferation of SMC
(Lindsey et al., 1983)

<table>
<thead>
<tr>
<th>Primary Treatment</th>
<th>Cell Number&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SEM</td>
</tr>
<tr>
<td><strong>A.</strong> Media</td>
<td>32,100±2,340</td>
</tr>
<tr>
<td>&quot; &quot; + 0.01 uM Cyc-A</td>
<td>27,000±1,730</td>
</tr>
<tr>
<td>&quot; &quot; + 1.0 uM Cyc-A</td>
<td>22,700±1,330</td>
</tr>
<tr>
<td>&quot; &quot; + 10 uM Cyc-A</td>
<td>No cells</td>
</tr>
<tr>
<td>1 uM Indomethacin</td>
<td>14,800±908</td>
</tr>
<tr>
<td>&quot; &quot; + 0.005 uM Cyc-A</td>
<td>15,700±807</td>
</tr>
<tr>
<td>&quot; &quot; + 0.01 uM Cyc-A</td>
<td>15,600±782</td>
</tr>
<tr>
<td>&quot; &quot; + 0.02 uM Cyc-A</td>
<td>18,000±1,050</td>
</tr>
<tr>
<td>1 uM Indomethacin</td>
<td>15,000±1,130</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.005 uM Cyc-A</td>
<td>13,000±1,130</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.01 uM Cyc-A</td>
<td>13,000±1,500</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.02 uM Cyc-A</td>
<td>13,200±590</td>
</tr>
<tr>
<td>1 uM Indomethacin</td>
<td>18,300±525</td>
</tr>
<tr>
<td>&quot; &quot; + 0.005 uM Cyc-A</td>
<td>20,000±457</td>
</tr>
<tr>
<td>&quot; &quot; + 0.01 uM Cyc-A</td>
<td>19,700±605</td>
</tr>
<tr>
<td>&quot; &quot; + 0.02 uM Cyc-A</td>
<td>19,800±312</td>
</tr>
<tr>
<td>&quot; &quot; + 0.05 uM Cyc-A</td>
<td>21,800±772</td>
</tr>
</tbody>
</table>

-continued-
Table 5. Effect of CsA on the Proliferation of SMC
(Lindsey et al., 1983) -continued-

<table>
<thead>
<tr>
<th>Primary Treatment</th>
<th>Cell Number&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. 10 uM Indomethacin</td>
<td>18,000+ 396</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.005 uM Cyc-A</td>
<td>19,000+ 660</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.01 uM Cyc-A</td>
<td>19,300+ 410</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.02 uM Cyc-A</td>
<td>19,200+ 559</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.05 uM Cyc-A</td>
<td>18,300+ 755</td>
</tr>
<tr>
<td>D. Media</td>
<td>5,470+ 554</td>
</tr>
<tr>
<td>&quot; &quot; + 0.005 uM Cyc-A</td>
<td>6,830+ 544</td>
</tr>
<tr>
<td>&quot; &quot; + 0.01 uM Cyc-A</td>
<td>7,830+ 506 P&lt;0.01</td>
</tr>
<tr>
<td>&quot; &quot; + 0.02 uM Cyc-A</td>
<td>6,220+ 196</td>
</tr>
<tr>
<td>60 uM 20:4(n-6)</td>
<td>3,490+ 215</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.005 uM Cyc-A</td>
<td>3,030+ 243</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.01 uM Cyc-A</td>
<td>2,940+ 298</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; + 0.02 uM Cyc-A</td>
<td>3,200+ 201</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values represent averaged values of arbitrary area units, n=6.
The effect of CsA on PGI$_2$ synthesis in proliferating SMC, cultures initially seeded at very low density, was also determined. Previous studies had shown that in proliferating cultures, reduced concentrations of the experimental agent were necessary to avoid toxicity (Huttner et al., 1977b; Cornwell et al., 1979; Morisaki et al., 1982a; 1982b; Morisaki et al., 1983). Preliminary work with CsA showed this to be true; therefore lower CsA concentrations were tested in proliferating cultures. As with confluent cells, CsA effectively stimulated PGI$_2$ synthesis in the proliferating cultures. A trend analysis showed a highly significant correlation between CsA concentration and PGI$_2$ (measured as 6-keto-PGF$_{1\alpha}$) yield (F-ratio 40.615) (Table 4).

CsA AND SMC PROLIFERATION

Previous studies found proliferation of SMC was promoted in the presence of either low concentrations of exogenous 20:4(n-6) or following treatment with agents that stimulate release of endogenous 20:4(n-6) (Cornwell and Morisaki, 1983; Huttner et al., 1977b; Morisaki et al., 1983). Because CsA appeared to stimulate release of endogenous substrate, it was tested in proliferating SMC cultures. As previously discussed, CsA inhibited proliferation at higher concentrations (0.1-10 uM) with obvious cell death occurring at 10 uM CsA (Table 5; Primary A). At lowered concentrations (0.005-0.02 uM) CsA promoted cell proliferation (Table 5; Primaries B,C,D). This effect was inhibited
with concurrent addition of 1 uM IM (Table 5; Primary B) and 10 uM IM (Table 5; Primary C). Addition of IM alone to cultures did not inhibit cell proliferation. These studies indicated CsA stimulates cell proliferation through prostanoid synthesis. Additionally, the presence of exogenous 20:4(n-6) inhibits cell proliferation. CsA does not overcome this inhibition (Table 5; Primary D). Exogenous 20:4 overwhelms endogenous release by CsA causing excessive prostaglandin and lipid peroxide formation which are inhibitory to SMC proliferation (Cornwell and Morisaki, 1983; Morisaki et al., 1982b; Morisaki et al., 1983).

As previously mentioned, CsA increased PGI₂ synthesis in proliferating cultures. A trend analysis was done to find if increased PGI₂ was merely a reflection of increased cell number. If this was true, the ratio of PGI₂ to cell number would not show a significant trend. The same experiment yielded the data shown for prostanoid synthesis (Table 4) and cell number (Table 5; Primary C) in proliferating cells. The amount of PGI₂ for each plate was divided by the number of each plate. Trend analysis found the ratio increased significantly with increasing CsA concentrations (ratio 4.735, P 0.04). This indicated that increased PGI₂ synthesis did not merely result due to increased cell number.
CsA AND PGE₂ BIOSYNTHESIS IN MØ

The stimulatory effect of CsA on SMC prompted studies investigating the effects on this compound on prostanoid synthesis of human peripheral blood MØ; cells directly involved with the immune response. MØ at 1 x 10⁶ cells/ml were incubated with varying concentrations of CsA for 48 hours. Media from cultures were removed, centrifuged to remove any cells and analyzed by RIA to determine PGE₂ levels. CsA increased PGE₂ biosynthesis in a dose dependent manner from 0.5 μM - 2.5 μM. PGE₂ levels plateaued from 2.5 - 20 μM CsA (Figure 4).

Viabilities and recoveries of MØ incubated for 48 and 72 hours at similar concentrations of CsA were determined in order to assess possible CsA cytotoxicity (Figure 5). Viability, based on trypan blue exclusion, and recovery slightly decreased at higher CsA concentrations at both 48 hours and (A) and 72 hours (B). Thus, at lower CsA concentrations, at which CsA increases PGE₂, there was no toxicity. The reduction in viability and recovery may account for the plateau at higher CsA concentrations.

THE KINETICS OF PGE₂ FORMATION BY CsA

The kinetics of PGE₂ formation by CsA were determined by treating MØ at 1 x 10⁶ cells/ml for various periods of time, with 1 ug/ml and 2.5 ug/ml CsA. PGE₂ levels in these cultures were compared to
Figure 4. PGE$_2$ Biosynthesis by MØ Following 48 Hour Incubation with Varying Concentrations of CsA. Results are Expressed as Mean ± S.E.M. for Three Experiments.
Figure 5. Viabilities (©-©) and Recoveries (○-○) of MØ following incubation with varying concentrations of CsA for 48 hours and 72 hours, A and B respectively. Results are for three experiments.
spontaneous PGE\textsubscript{2} release of MØ incubated concurrently in media alone (Figure 7). As expected, there is an increase of PGE\textsubscript{2} levels beyond control in both 1.0 and 2.5 ug/ml cultures. PGE\textsubscript{2} levels begin to increase within 12 hours and maximize between 24 and 48 hours. At both concentrations of CsA, PGE\textsubscript{2} levels dropped off by approximately 30% between 48 and 72 hours. This was possibly due to the presence of enzymes capable of degrading PGE\textsubscript{2} such as 15-hydroxy prostaglandin dehydrogenase (Wong et al., 1982).

THE INABILITY OF CsA TO INCREASE PGE\textsubscript{2} BIOSYNTHESIS IN THE PRESENCE OF IM AND EXOGENOUS 20:4(n-6)

MØ were incubated for 48 hours at various CsA concentrations with and without 10 uM IM. IM effectively inhibited PGE\textsubscript{2} biosynthesis at all CsA concentrations tested to levels less than 1% (Table 6).

CsA enhances PGE\textsubscript{2} biosynthesis in MØ by stimulating either release of substrate from phospholipid or enzymatic conversion of 20:4(n-6) to prostanoid. As previously mentioned, addition of 20:4(n-6) to cultures circumvents substrate release. Therefore, stimulation of this step by an agent would be abolished in the presence of exogenous 20:4(n-6). MØ at 1 x 10\textsuperscript{6} cells/ml were incubated with 0, 18 or 45 uM 20:4(n-6) either alone or supplemented with 2.5 or 10 ug/ml CsA. As expected, PGE\textsubscript{2} levels increased with 18 and 45 uM 20:4(n-6). However, concurrent treatment with either 2.5 or 10 ug/ml CsA did not enhance PGE\textsubscript{2} levels beyond those of 20:4(n-6) alone (Figure 7).
Figure 6. Stimulation of PGE$_2$ Biosynthesis of MØ by CsA Over Varying Periods of Time. Results Represent Mean ± S.E.M. for Four Experiments.
Table 6. PGE$_2$ Biosynthesis in MØ with CsA in the Presence or Absence of IM (Whisler et. al., 1984).

<table>
<thead>
<tr>
<th>CsA concentration (ug/ml)</th>
<th>PGE Levels (pg/10$^6$MØ)$^a$</th>
<th>Percentage$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Indo</td>
<td>+Indo</td>
</tr>
<tr>
<td>0</td>
<td>9,438±2,513</td>
<td>89±12</td>
</tr>
<tr>
<td>5</td>
<td>20,150±4,698</td>
<td>134±39</td>
</tr>
<tr>
<td>10</td>
<td>23,411±5,807</td>
<td>153±25</td>
</tr>
<tr>
<td>15</td>
<td>22,976±6,899</td>
<td>107±34</td>
</tr>
</tbody>
</table>

$^a$MØ incubated for 48 hours with indicated CsA concentrations and/or 10 uM IM. Results expressed as mean ± S.E.M.

$^b$Percentage equals PGE$_2$ levels of MØ incubated in the presence of IM divided by PGE$_2$ levels of MØ incubated in the absence of IM(X100).
Figure 7. PGE$_2$ Biosynthesis of MØ After 48 Hour Incubation with CsA and/or Exogenous 20:4(n-6). Results Represent Mean ± S.E.M. for Three Experiments.
CsA AND HLA-DR EXPRESSION BY MØ

The presence of HLA-DR antigens on the cell surface of the MØ is a critical accessory function of MØ. The effect of CsA on this phenomena was investigated by incubating MØ either alone or with varying concentrations of CsA. Another series of cultures were treated similarly but were supplemented with 10 uM IM. After 48 hour incubation, the MØ were removed from the media and expression of membrane HLA-DR was measured by exposing MØ to fluorescent antibodies directed against the HLA-DR molecules. The density of these antibodies bound to the cell surface was quantitated by FMF. 10,000 cells were analyzed for fluorescent intensities and visually expressed as a distribution profile with number of cells plotted vs. fluorescent intensity. A shift to the left indicates down regulation of HLA-DR expression. Increasing concentrations effectively inhibited HLA-DR expression as seen with the profiles shown in Figure 8. This inhibition of HLA-DR expression did not occur in the presence of IM at CsA concentrations 0.6 - 6 ug/ml CsA. IM did not effectively prevent inhibition of HLA-DR expression by CsA at the higher concentrations 12 and 24 ug/ml CsA.
Figure 8. HLA-DR Expression of MØ Following 48 Hour Incubation with Varying Concentrations of CsA and/or IM. Results are for One Representative Experiment of Five.
PGE\(_2\) and HLA-DR EXPRESSION BY MØ

The protective effect of concurrent addition of IM to CsA treated cultures indicated the possible involvement of prostanoids in reduction of HLA-DR. MØ were incubated with 10 \(\mu\)M IM, to inhibit endogenous prostanoid synthesis, with and without varying concentrations of PGE\(_2\). Following 48 hour incubation, HLA-DR expression was quantitated by FMF. PGE\(_2\) progressively reduced HLA-DR expression at 60 \(\mu\)M to 0.3 \(\mu\)M (Figure 9).

EFFECTS OF CsA ON OTHER PHYSICAL CHARACTERISTICS OF MØ

Reduction of HLA-DR expression at 12 and 24 \(\mu\)g/ml CsA was not protected by IM. This indicated the higher CsA concentrations were diminishing antigen expression by other effects. Possible perturbations of the membrane and depletion of MØ subsets were determined by investigating indirect light scatter and channalyzer analysis of MØ following CsA treatment. Indirect light scattering properties would be altered with changing membrane characteristics. Indirect light scatter profiles of MØ treated with varying concentrations of CsA with or without 10 \(\mu\)M IM for 48 hours showed that CsA concentrations greater than 0.5 \(\mu\)g/ml were reduced either with or without IM. This effect was most apparent at 6, 12 and 24 \(\mu\)g/ml CsA (Figure 10). Channelyzer analysis of MØ treated at 6, 12 and 24 \(\mu\)g/ml CsA with or without IM
Figure 9. Reduction of HLA-DR Expression of MØ After 48 Hour Incubation with Varying Concentrations of PGE₂. Results are for One Representative Experiment of Four.
Figure 10. Effects of Various Concentrations of CsA and/or IM on the Indirect Light Scattering Properties of MØ. The Results are Representative of Three Experiments.
showed that CsA diminished those MØ subsets with relatively greater cellular volumes. This effect occurred in the presence or absence of IM (Figure 11).

EFFECT OF MØ OR MØM ON PGI₂ BIOSYNTHESIS OF CONFLUENT SMC

The ability of MØ or MØM to stimulate PGI₂ synthesis, measured by 6-keto PGF₁α, was tested using confluent SMC cultures. 2 x 10⁶ MØ were added directly to cultures, incubated for 24 hours and 6-keto PGF₁α levels were measured. The results of five different experiments are shown in Table 7. The addition of MØ caused a significant increase in 6-keto PGF₁α as determined by a paired T-test comparing the significance of difference between control and MØ treated cultures. This increase is not due to PGI₂ synthesis by MØ since MØ maintained in media in the absence of SMC contained only 0.004 nmol/culture 6-keto PGF₁α.

The ability of MØM to effect PGI₂ synthesis of SMC was determined in a similar fashion. MØM was obtained after 24 hour incubation of MØ at 2 x 10⁶ cells/ml. Unlike the previous experiments, addition of MØM did not significantly effect PGI₂ biosynthesis in SMC (Table 8). Again, 6-keto PGF₁α levels in MØM cultures in the absence of SMC only contained 0.004 nmol/culture 6-keto PGF₁α. These results indicate that MØM does not stimulate prostanoid synthesis in SMC.
Figure 11. Distribution Profiles of MØ Cellular Volumes After Exposure to Varying Concentrations of CsA and/or IM. The Profiles Represent MØ Preincubated in: A) Medium Alone, B) Medium + IM, C) Medium + 6 ug/ml CsA, D) Medium + 6 ug/ml CsA + IM, E) Medium + 12 ug/ml CsA, F) Medium + 12 ug/ml CsA + IM, G) Medium + 24 ug/ml CsA, and H) Medium + 24 ug/ml CsA + IM.
Table 7. Effect of MØ on PGI2 (6-keto PGF₁α)

Synthesis of Confluent SMC

<table>
<thead>
<tr>
<th>Primary</th>
<th>Treatment</th>
<th>6-keto PGF₁α&lt;sub&gt;a,b&lt;/sub&gt; (nmol culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>media</td>
<td>0.548</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; + MØC</td>
<td>0.862</td>
</tr>
<tr>
<td>B</td>
<td>media</td>
<td>0.707</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; + MØC</td>
<td>1.285</td>
</tr>
<tr>
<td>C</td>
<td>media</td>
<td>0.406</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; + MØC</td>
<td>0.568</td>
</tr>
<tr>
<td>D</td>
<td>media</td>
<td>0.627</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; + MØC</td>
<td>1.437</td>
</tr>
<tr>
<td>E</td>
<td>media</td>
<td>0.656</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; + MØC</td>
<td>1.028</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values represent average of duplicate samples

<sup>b</sup>Differences between two treatment groups were determined to be significant based on a paired t-test P<0.01 (t=3.75, d.f. = 4).

<sup>c</sup>1 ml of media containing 2 x 10⁶ MØ/ml was added to 3 ml of experimental media.
Table 8. Effect of M\(\text{M}\) on PGI\(_2\) (6-keto PGF\(_{1\alpha}\))

Synthesis of Confluent SMC

<table>
<thead>
<tr>
<th>Primary</th>
<th>Treatment</th>
<th>6-keto PGF(_{1\alpha}) (nmol/culture)(^{a,b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>media</td>
<td>0.124</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; + M(\text{M})(_{c,d})</td>
<td>0.127</td>
</tr>
<tr>
<td>G</td>
<td>media</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; + M(\text{M})(_{c,d})</td>
<td>1.27</td>
</tr>
<tr>
<td>H</td>
<td>media</td>
<td>0.761</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; + M(\text{M})(_{c,d})</td>
<td>0.447</td>
</tr>
<tr>
<td>I</td>
<td>media</td>
<td>0.369</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; + M(\text{M})(_{c,d})</td>
<td>0.480</td>
</tr>
</tbody>
</table>

\(^{a}\)Values represent average of duplicate samples

\(^{b}\)Differences between two treatment groups were determined to be not significant based on paired t-test (t=1.43 d.f. = 3)

\(^{c}\)2 \(\times 10^6\) M\(\text{M}\)/ml were incubated for 24 hours. Media was centrifuged to remove cells and 1 ml of M\(\text{M}\) added to 3 ml of experimental media.

\(^{d}\)avg [6-keto PGF\(_{1\alpha}\)] in M\(\text{M}\) = 0.004 ng/culture.
MØ-CONDITIONED MEDIA AND SMC PROLIFERATION

The effects of MØ-conditioned media (MØM) on smooth muscle cell proliferation has been previously discussed. The following studies confirmed the presence of growth factor(s) released from cultured MØ which stimulated SMC proliferation.

Optimum MØ Concentration and Incubation Time for MØM

MØ were cultured at varying concentrations and for various incubation times to determine optimal conditioning parameters for MØM. SMC cultures were treated with MØM from MØ cultures at 1 x 10^6 cells/ml incubated for varying times. Following incubation, MØ were removed by centrifugation. Proliferating SMC were treated with MØM as described. Significant stimulation occurred at 12 hours, reaching a maximum at 24 hours (Table 9). Similarly, MØM at 0.5, 1 and 2 x 10^6 cells/ml were cultured in media for 24 hours. The largest increase in SMC growth occurred with MØM from MØ cultures at 2 x 10^6 cells/ml (Table 10).

Effect of MØM on SMC Proliferation and PGE₂ Synthesis

The mitogenic factor present in MØM is considered to be a protein called Monocyte Derived Growth Factor (MØDGF). The role of prostanoids synthesized by MØ as growth promoters had not previously
Table 9. Effect of Different MØM, Prepared By Varying Incubation Time, On SMC Proliferation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Growth (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 3(6)</td>
</tr>
<tr>
<td>&quot; &quot; + MØM (6 hrs)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>139 ± 6(6)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot; &quot; + MØM (12 hrs)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>156 ± 8(6)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot; &quot; + MØM (24 hrs)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>214 ± 15(6)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot; &quot; + MØM (48 hrs)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>184 ± 10(6)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot; &quot; + MØM (72 hrs)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>186 ± 9(6)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>MØ at 1 x 10⁶ cells/ml were incubated for indicated period of time

<sup>b</sup>Mean ± S.E.M.; number of experiments in parenthesis

<sup>c</sup>Differed significantly from Control (Tukey-HSD test)
Table 10. Effect of Different MØM, Prepared by Varying MØ Concentrations, on SMC Proliferation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Growth (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 3(6)</td>
</tr>
<tr>
<td>&quot; &quot; &quot; + MØM (0.5 x 10&lt;sup&gt;6&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>117 ± 1(6)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot; &quot; &quot; + MØM (1 x 10&lt;sup&gt;6&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116 ± 3(6)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot; &quot; &quot; + MØM (2 x 10&lt;sup&gt;6&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>160 ± 4(6)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration of MØ used to condition media
<sup>b</sup> Mean ± S.E.M.; number of experiments in parenthesis
<sup>c</sup> Differed significantly from Control (Tukey-HSD test)
been reported. MØM was added to proliferating SMC in the presence and absence of 10 uM IM. Control cultures were treated similarly. As expected, MØM effectively stimulated SMC proliferation (Table 11). Treatment of SMC with 10 uM IM alone did significantly decrease proliferation and PGE₂ production. However, concurrent addition of MØM and 10 uM IM did not reverse the stimulatory effect of MØM. PGE₂ levels did differ slightly between MØM and MØM+IM cultures (Table 11). These studies indicated that addition of MØM does not stimulate PGE₂ synthesis in SMC. Higher PGE₂ levels in MØM treated cultures probably reflected PGE₂ produced by both MØ and SMC.

MØ were conditioned as previously described, in the presence of 10 uM IM. MØM IM, media removed from MØ conditioned in the presence of 10 uM IM stimulated SMC proliferation also. However, this effect was significantly less than MØM treated cultures. Also, PGE₂ levels of MØM IM cultures were significantly reduced. This indicated conditioning MØ with 10 uM IM effectively inhibited PGE₂ synthesis by MØ and reduced the amount of prostanoid added to SMC cultures.
Table 11. Effect of MØM on PGE$_2$ Synthesis and Proliferation with SMC

<table>
<thead>
<tr>
<th>Treatment$^a$</th>
<th>Cell Number$^a$</th>
<th>PGE$_2$$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>100 $\pm$ 2.9 (25)</td>
<td>7,620 $\pm$ 538 (6)</td>
</tr>
<tr>
<td>Control + IM$^c$</td>
<td>86.8 $\pm$ 2.8 (26)</td>
<td>725 $\pm$ 31 (6)</td>
</tr>
<tr>
<td></td>
<td>P$&lt;0.005$</td>
<td>P$&lt;0.005$</td>
</tr>
<tr>
<td>2. Control + MØM$^d$</td>
<td>166 $\pm$ 4.2 (12)</td>
<td>45,600 $\pm$ 620 (6)</td>
</tr>
<tr>
<td>Control + MØM$^d$ + IM$^c$</td>
<td>159 $\pm$ 6.7 (12)</td>
<td>32,700 $\pm$ 1,310 (6)</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>P$&lt;0.005$</td>
</tr>
<tr>
<td>3. Control + MØM$^d$</td>
<td>179 $\pm$ 5.2 (15)</td>
<td>70,000 $\pm$ 1,590 (6)</td>
</tr>
<tr>
<td>Control + MØM$^d$ + IM$^e$</td>
<td>153 $\pm$ 6.3 (15)</td>
<td>8,750 $\pm$ 439 (6)</td>
</tr>
<tr>
<td></td>
<td>P$&lt;0.005$</td>
<td>P$&lt;0.005$</td>
</tr>
</tbody>
</table>

$^a$Arbitrary units (normalized); mean $\pm$ SEM; number of experiments in parentheses.

$^b$Prostanoid in pg/culture; mean $\pm$ SEM; number of experiments in parentheses.

$^c$Indomethacin (IM, 10uM) added to proliferating SMC cultures.

$^d$Media preincubated for 24 hour with MØ (2 x 10$^6$ cells/ml).

$^e$Media containing 10 uM preincubated for 24 h with MØ (2 x 10$^6$ cells/ml). Levels of significance determined by an unpaired t-test.
MØ inhibit or promote cell mediated immunity through release of soluble mediators such as prostanoids or IL-1 and by expression of HLA-DR antigens (Unanue, 1981). Prostanoids are considered inhibitors of T-cell transformation and proliferation, a key step in immune responses, such as rejection of transplanted organs (Lomnitzer et al., 1976; Smith et al., 1971; Mihas, 1975; Goodwin et al., 1977; Bockman and Rothschild, 1979; Rappaport and Dodge, 1980). Prostanoids have, in fact, been used to prevent allograft rejection (Mertin, 1976; McHugh et al., 1977; Anderson et al., 1976; 1977; Strom, 1976; Kakita et al., 1975). Expression of the cell surface HLA-DR antigens by MØ is a vital accessory function. Sufficient quantities of HLA-DR must be presented by MØ to T-cell in order for initial recognition and subsequent activation to occur (Unanue, 1981; Stobo, 1982).

Currently, the details of the mode of action of the immunosuppressant, CsA, are unclear. However, it is accepted that CsA inhibits early stages of T-cell activation and proliferation. Vital elements of T-cell activation and proliferation include; MØ release of IL-1, MØ expression of HLA-DR and T-cell release of IL-2 (Lafferty et al., 1980; 1983; Andrus and Lafferty, 1982). Studies have shown CsA
inhibits both IL-1 and IL-2 production (Bunjes et al., 1981; Tosatos et al., 1982; Andrus and Lafferty, 1982; Dos Reis and Shevach, 1982). The effects of CsA on the remaining two elements, MØ prostanoid synthesis and HLA-DR expression are considered in this dissertation.

CsA promoted prostanoid synthesis in both somatic, SMC, and immunocompetent, MØ, cell types. The initial SMC studies found CsA increased PGI$_2$ synthesis in both proliferating and confluent cultures (Table 4). CsA also stimulated PGE$_2$ synthesis and release by human peripheral blood MØ at concentrations that did not alter viability and recovery (Figures 4 and 5). The CsA effect was abolished in the presence of IM (Table 6). CsA increased PGE$_2$ levels significantly beyond spontaneous PGE$_2$ release after incubation of 12 to 24 hours (Figure 6). The PGE$_2$ levels obtained following CsA are within the range suggested by Goodwin et al. as adequate for inhibition of mitogen induced lymphocyte transformation (1 x 10$^{-7}$ - 1 x 10$^{-8}$M) (Goodwin et al., 1977). These findings are supported by a recent article which also examined prostanoid synthesis by MØ following CsA treatment. Schultz et al. also found CsA increased prostanoid levels in both unstimulated and mitogen and alloantigen-activated MØ (Schultz et al., 1984).

In both SMC and MØ conditioned cultures, CsA did not stimulate prostanoid synthesis in the presence of exogenous 20:4(n-6) (Table 5 and Figure 7). These studies indicated that CsA potentiates substrate release as opposed to stimulating enzymatic conversion of 20:4(n-6) to PGI$_2$ or PGE$_2$. Additionally, CsA promoted SMC proliferation, an
effect previously found with other agents that stimulate endogenous
20:4(n-6) release (Huttner et al., 1977b; Morisaki et al., 1983;
Cornwell and Morisaki, 1984).

Preliminary results from studies recently conducted by Kaseki,
indicate CsA stimulated release of 14C-20:4(n-6) from prelabeled SMC.
Cultures treated with CsA in the presence or absence of IM released
similar amounts of radioactivity into media. HPLC analysis of the media
following ethyl acetate extraction, revealed CsA stimulated accumulation
of 14C-20:4(n-6). In CsA and IM cultures this accumulation was
increased. Comparable results were demonstrated with A23187, a com-
monly accepted releasing agent (Kaseki et al., unpublished observations).

This dissertation does not include functional studies of CsA
treated cells in connection with prostanoid synthesis. However, two re-
ports which examine the effects of CsA on T-cell mitogenesis suggest CsA
acts through prostanoids. Foa and Catovsky found CsA at 1-10 ug/ml
inhibited formation of T-cell colonies in soft agar cultures. In paral-
lel, Bockman and Rothschild used the identical technique in their study
of prostanoid effects on T-cell colony formation. Exogenous PGE2 de-
creased T-cell colony formation. Also, they reported addition of low
numbers of MØ mimicked the effects of exogenous PGE2. Concurrent
treatment of MØ and IM did not prevent colony formation (Bockman and
Rothschild, 1979). These studies indicated a possible role of
prostanoids in the mechanism of action of CsA.

A second accessory function of MØ was investigated in this dis-
sertation; the expression of HLA-DR antigens by MØ. As previously
discussed, these antigens must be present on the MØ cell surface in order for initial T-cell activation. CsA effectively inhibited the appearance of HLA-DR on MØ at concentrations previously used to promote PGE\textsubscript{2} synthesis. The presence of IM protected antigen expression at lower CsA levels (Figure 8). Addition of PGE\textsubscript{2} mimicked CsA effects and reduced the amount of antigen on MØ cell surface (Figure 9). Previous studies support these findings. Yem et al. found endotoxin and zymosin treated MØ expressed reduced amounts of antigen (Yem et al., 1981). Both of these stimuli promote prostaglandin synthesis in MØ (Glenn and Ross, 1981; Kurland and Bockman, 1978). Also, Snyder et al. found exogenous PGE\textsubscript{1} and PGE\textsubscript{2} reduced Ia antigen, the HLA-DR equivalent, expression by murine peritoneal MØ (Snyder et al., 1982).

At higher CsA concentrations, IM did not protect HLA-DR expression. Channalyzer results indicated a loss of MØ with greater volume at 6, 12 and 24 \textmu{g}/mL CsA with or without IM (Figure 11). Thus, reduced amounts of expression per cell HLA-DR could be the results of loss of these larger MØ. Light scatter analysis however suggests other effects of CsA on MØ. Indirect light scattering properties of a cell depend upon cell volume, shape and refractive indices of cellular membranes and internal structures (Salzman, 1982). A marked decrease in the indirect light scattering profiles occurred at all CsA concentrations tested with little alteration in the presence of IM. Changes in these profiles are more dramatic than channalyzer data. These studies suggest that CsA may alter or damage the cellular membrane of MØ at higher concentrations. Therefore, CsA may reduce HLA-DR expression by perturbing the lipid bilayer of membrane.
Thus, the results of this dissertation suggest CsA exerts immunosuppressive effects by altering accessory cell functions of MØ. This agent increased prostanoid production and reduced HLA-DR expression, two events which in combination result in inhibition of T-cell transformation and ultimate immunosuppression.

Numerous studies have found tissue MØ release a MØ derived growth factor (Leibovich and Ross, 1976; Unanue and Kiely, 1977; Wall et al., 1978; Greenburg and Hunt, 1978; Goldman and Bai-Shavit, 1979; Jalkanen et al., 1979, Wahl et al., 1979, Martin et al., 1980). Two previous reports examined the ability of human peripheral blood MØ to produce a growth factor. Ziats and Robertson found a MØDGF from peripheral blood MØ stimulated long term cultures of proliferating human aortal smooth muscle cells. They used both unstimulated MØ and MØ stimulated with zymosin to condition treatment media. By Day 3 of culture both MØM and MØMZY had increased cell growth. By Day 6 this effect was significantly obvious. Cell number was determined by Coulter Counter analysis (Ziats and Robertson, 1981). These results contrast however with another study investigating MØDGF from human peripheral blood MØ. Glenn and Ross measured ³H-thymidine incorporation by 3T3 cells, human smooth muscle and fibroblast cells following 19 hour incubation with media conditioned with fresh and cultured peripheral MØ. They found the MØ produced sufficient MØDGF only after five to seven days in culture (Glenn and Ross, 1981).
This dissertation examined the effect of human peripheral MØ and MØ conditioned media on prostanoid synthesis by SMC as well as the effect of MØM on SMC proliferation. Studies with confluent SMC treated with $2 \times 10^6$ MØ found significant increases in PGI$_2$ levels (Table 7). MØ did not synthesize appreciable amounts of PGI$_2$ (0.004 nmol/culture) consequently PGI$_2$ levels reflect prostanoid synthesis by SMC. Addition of MØM did not increase PGI$_2$ levels in media from treated SMC cultures (Table 8). Thus, MØ must be present for increased prostanoid synthesis by SMC. This effect could be due to increased availability of 20:4(n-6), released from MØ, for utilization by SMC.

The studies in this dissertation using a similar experimental design, support the results found by Ziats and Robertson. The addition of MØM to proliferating SMC cultures enhanced growth (Table 11). Optimum MØ concentration and incubation time for MØM were determined to be $2 \times 10^6$ cells/ml and 24 hours respectively (Tables 9 and 10). SMC proliferation was not diminished by concurrent addition of IM with MØM. However, treatment of SMC with MØM, media obtained from MØ conditioned in the presence of IM, did reduce proliferation in comparison to MØM treated cultures (Table 11).

PGE$_2$ levels were measured in media removed from cultures prior to staining. Since both SMC and MØ synthesize PGE$_2$, the levels in MØM treated SMC cultures reflect PGE$_2$ produced by both cell types. PGE$_2$ levels were greatly decreased in SMC cultures treated with IM alone. Supplementation with MØM significantly increased PGE$_2$ and
concurrent addition of IM with MØM reduced this effect slightly. However, PGE$_2$ levels in MØM+IM cultures were still significantly higher than control cultures. This effect is probably due to increased number of cells present in these cultures since, as previously discussed, SMC proliferation was not significantly decreased with MØM and IM treatment. Conditioning of MØ in the presence of IM inhibited PGE$_2$ synthesis by MØ. This is reflected by the substantial decrease in total PGE$_2$ levels of the cultures.

Three factors may cause enhanced SMC proliferation by MØM: the presence if a MØDGF; increased levels of PGE$_2$; and/or stimulated release of endogenous substrate. Because MØM does not stimulate PGI$_2$ synthesis in confluent SMC, it seems unlikely that MØM enhances substrate release. MØM contains sufficient levels of PGE$_2$ that, when added to SMC cultures, may act as exogenous PGE$_2$ and stimulate SMC growth. Previous studies have shown addition of low levels of exogenous prostanoids promoted SMC proliferation (Cornwell et al., 1979; 1984; Cornwell and Morisaki, 1984 and Huttner et al., 1977b). This is indicated by the results obtained when MØ were incubated in the presence of IM (MØM$_{IM}$). PGE$_2$ levels of MØM$_{IM}$ were reduced and SMC growth was not enhanced to the same extent as MØM treated cultures. However, proliferation of MØM$_{IM}$ treated cultures was significantly promoted beyond control. Thus, enhancement of growth is not totally due to PGE$_2$ but, as predicted, the presence of a MØDGF. These studies suggest synergistic effects of MØDGF and prostanoid synthesized and released by MØ on cell growth.
REFERENCES


